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Effects of lactic acid bacteria isolated from fermented mustard on lowering cholesterol

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PEER REVIEW

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Comments

This work shows strain B0006, B0007 and B0022 with remarkable cholesterol-lowering activity are acid and bile salt tolerance. In particular, strain B0007 and B0022 displayed strong adherence to Caco-2 cells. Considering the adherent activity, we suggest that strains B0007 and B0022 may be potential alternatives for supplement to lower serum cholesterol. Details on Page 527

ABSTRACT

Objective: To evaluate the ability of lactic acid bacteria (LAB) strains isolated from fermented mustard to lower the cholesterol *in vitro*.

Methods: The ability of 50 LAB strains isolated from fermented mustard on lowering cholesterol *in vitro* was determined by modified o-phtshalaldehyde method. The LAB isolates were analyzed for their resistance to acid and bile salt. Strains with lowering cholesterol activity, were determined adherence to Caco-2 cells.

Results: Strain B0007, B0006 and B0022 assimilated more cholesterol than BCRC10474 and BCRC 17010. The isolated strains showed tolerance to pH 3.0 for 3 h despite variations in the degree of viability and bile-tolerant strains, with more than 10⁸ CFU/mL after incubation for 24 h at 1% oxigall in MRS. In addition, strain B0007 and B0022 identified as *Lactobacillus plantarum* with 16S rDNA sequences were able to adhere to the Caco-2 cell lines.

Conclusions: These strains B0007 and B0022 may be potential functional sources for cholesterol-lowering activities as well as adhering to Caco-2 cell lines.

KEYWORDS

Cholesterol-lowering activity, Probiotic, Lactic acid bacteria, Acid, Bile tolerance

1. Introduction

Overmuch cholesterol in the blood and diet is a major risk factor for coronary heart disease and colon cancer[1]. For each 1 mmol above the normal cholesterol level, the risk of coronary heart disease was approximately 35% higher and coronary death was 45% higher[2]. Recently, lactic

acid bacteria (LAB) have attracted attention as potential cholesterol-lowering agent[3]. Consumption of dairy products containing probiotics has been proposed as a means to lower serum cholesterol[4]. Several studies indicated consumption of certain cultured dairy products could lower total plasma cholesterol and low-density lipoprotein cholesterol[1,5]. The reduction of serum cholesterol could be an important health

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benefit of LAB, demonstrated in human, mouse, pig studies and rats^[5–7]. Therefore, the investigation of effective natural ingredients from food that could decrease concentrations of serum cholesterol has recently drawn a great deal of attention^[3]. Many kinds of *Lactobacillus* cultures exerted potential hypocholesterolemic activity^[5,8].

Fermented mustard (picked mustard green) is made from green mustard and its production is a spontaneous fermentation process by a mixed microbial population mainly composed of LAB. The aim of this study was to screen lactobacilli with probiotic characteristics isolated from traditionally fermented mustard, and to determine the effect of the screened *Lactobacillus* strains for their ability to lower cholesterol and the strains were also identified.

2. Materials and methods

2.1. Traditional Taiwan fermented mustard samples

The liquor samples of fermented mustard were collected from the farms of central and southern Taiwan.

2.2. Isolation of LAB

The diluted liquor samples were spread on the surface of MRS agar containing 5 g/L calcium carbonate and then incubated at 37 °C for 1–2 d. Colonies of clear zones on MRS agar plates were randomly selected and purified. Only Gram-positive and catalase-negative strains were taken as presumptive LAB and stored at 4 °C in MRS agar plate.

2.3. Cholesterol removal

The cholesterol removal was performed using procedures described by Wang *et al*^[1]. In brief, freshly prepared MRS broth was supplemented with 0.30% oxigall (Sigma, MO, USA) as a bile salt. Water-soluble cholesterol (polyoxyethylene cholesteryl sebacate) was filter-sterilized and added to the broth at a final concentration of 200 to 300 µg/mL, inoculated with each strain and incubated anaerobically at 37 °C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colorimetric method as described by Wang *et al*^[1]. The aliquot (100 µL) were added with 100 µL of KOH (33% w/v) and 200 µL of absolute ethanol, vortexed for 1 min, and heated at 37 °C for 15 min. After cooling, 200 µL of distilled water and 300 µL of hexane were added and vortexed for 1 min. The hexane layer (100 µL) was transferred into a glass tube and evaporated under nitrogen. The residue was immediately dissolved in 200 µL of *o*-phthalaldehyde reagent. After complete mixing, 50 µL of concentrated sulfuric acid were added and the mixture

was vortexed for 1 min. Absorbance was read at 540 nm with ELISA reader (Multiskan EX, LabSystem, Gyeongbuk, Korea) after 10 min. All experiments were replicated twice.

2.4. Cholesterol removal by dead and resting cells

Freshly prepared MRS broth containing 0.30% oxigall was inoculated with each strain of LAB and incubated at 37 °C for 20 h. Cells were harvested after the incubation period by centrifuging at 10000 r/min (Microspin 24, Sorvall Instruments, Melbourne, Australia) at 4 °C for 10 min. The cell pellet was washed twice with sterile distilled water. For preparation of heat-killed cells, the cell pellet was suspended in 10 mL of sterile distilled water and autoclaved for 15 min at 121 °C. For preparation of resting cells, the cell pellet was suspended in 10 mL of 0.05 mol/L sterile phosphate buffer (pH 6.8)^[9]. All samples were suspended in MRS broth containing 0.30% oxigall and water-soluble cholesterol and assayed for cholesterol content as mentioned above. The experiments were repeated twice.

2.5. Acid tolerance

Acid tolerance of the cultures was investigated by incubating the organisms in MRS broth supplemented with 0.30% oxigall. The pH was adjusted to 3.0 and 2.0 with HCl and cultures were incubated at 37 °C for 3 h. Each of the isolated LAB was subcultured at least 3 times before experimental use, followed by centrifugation after the final subculture, inoculation (10% v/v) into the broth, and growth monitoring using the plate count method^[9]. The experiments were repeated twice.

2.6. Bile tolerance

The LAB isolates were analyzed for their resistance to bile salt. The MRS broths at concentrations of 0%, 0.5% and 1.0% (w/v) of oxigall were prepared and dispensed in 10 mL volumes and sterilized by heating 121 °C for 15 min. Each of the isolated LAB was subcultured at least 3 times before experimental use, followed by centrifugation after the final subculture, inoculation (10% v/v) into the broth, and growth monitoring using the plate count method^[9]. The reaction mixture and MRS broth were incubated at 37 °C for 24 h. All the experiments were repeated twice.

2.7. Adhesion assay

The Caco-2 cell-lines were purchased from the Bioresources Collection and Research Center (BCRC), Hsin-Chu, Taiwan. Cells were grown routinely in Dulbecco's modified Eagle's minimal essential medium (DMEM; GIBCO BRL Laboratories, NY, USA) containing 1.0 mmol/L sodium

pyruvate, and supplemented with 10% (v/v) fetal bovine serum and 50 unit/mL penicillin–streptomycin (GIBCO). For adhesion assay, monolayers of Caco–2 cells were prepared on glass cover slips that were placed in six–well tissue–culture plates (NUNC products, supplied by Life Technologies, Auckland, New Zealand). Maintenance of cells and all experiments with cell–lines were carried out at 37 °C in an atmosphere of 5% CO₂. Prior to the adhesion test, all LAB were washed twice with phosphate buffered saline and centrifuged for 5 min at 2100 r/min. Bacterial cells were resuspended in 1 mL minimum essential medium. One hundred microliters of the suspension (1×10⁸ CFU/mL) was transferred to a washed monolayer of cells, and incubated for 2 h at 37 °C in 5% CO₂. Monolayers were washed four times with phosphate buffered saline (pH 7.4), fixed in methanol, Gram stained (Baxter Scientific Products, McGraw Park, Miami, FL, USA) and examined microscopically under an oil immersion lens. Numbers of LAB cells adhered to the cultured cell lines were counted according to the method of Gopal *et al.*[10].

2.8. Strains identification

LAB isolates that showed physiological tests as a probiotic, acid tolerance and bile tolerance and lowering cholesterol activity were identified by API 50 CHL fermentation assays (BioMerieux, S.A., Marcy l’Etoile, France) and 16S rDNA sequence analysis. The primarily confirmed by API 50 CHL fermentation assays were following the instruction procedure. In 16S rDNA sequence analysis, the PCR primers designed from the 16S rRNA genes primers 27F/1492F[11]. For the PCR assay, the method of Michael *et al.* was followed[11]. The amplification products were purified with DNA purification kit (Promega, Madison, WI, USA) and sequenced by Nucleic acid Synthesis and Analysis Core Laboratory (Cheng Kung University College of Medicine, Tainan, Taiwan). Sequence homologies were examined by comparing the obtained sequence with those in the DNA data bases (<http://www.ncbi.nlm.nih.gov/BLAST>)[12].

2.9. Statistical analysis

All data were recorded as mean±SD. Statistical analysis utilized the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan’s multiple range tests at a level of $P<0.05$.

3. Results

A total of 50 microbial colonies were obtained from fermented mustard. They were selected based on criteria

such as morphological shape, catalysis negativity, Gram positive, and lactic acid formation, consequently and were considered as LAB. Of the 50 strains, 11 strains indicated in Table 1 exhibited higher cholesterol assimilation than reference strains, BCRC 10747 and BCRC 17010. Apparently, cholesterol removal varied among strains and ranged from 110.69 µg/mL to 179.94 µg/mL. Especially, strain B0007, B0006 and B0022 showed remarkable cholesterol removal among the strains ($P<0.05$). Thus, strain B0007, B0006 and B0022 were selected to test acid and bile tolerance in the following tests.

Table 1

Assimilation of cholesterol by lactic acid bacteria.

Strains	Assimilation of cholesterol (µg/mL)
BCRC17010	79.03±5.01 ^b
BCRC17474	43.88±3.67 ^a
B0006	153.39±8.74 ^{de}
B0007	179.94±8.25 ^e
B0009	110.67±10.92 ^{bc}
B0010	113.09±5.53 ^{bc}
B0011	116.12±6.58 ^{de}
B0012	134.91±2.73 ^{cd}
B0015	134.61±31.37 ^{cd}
B0021	113.70±5.92 ^{bc}
B0022	154.00±7.27 ^{de}
B0023	131.27±2.73 ^{cd}
B0025	130.36±7.77 ^{cd}

BCRC17474: *Lactobacillus johnsonii* (*L. johnsonii*). BCRC17010: *Lactobacillus acidophilus* (*L. acidophilus*). The data values are expressed as the mean±SD ($n=3$). Values in a column with different superscript letters are significantly different ($P<0.05$).

Table 2 shows cholesterol removal by growing, resting and dead cells. The levels of cholesterol removal ranged from 1.40 to 35.44 µg/mL for resting cells and 0–54.21 µg/mL for dead cells. Apparently, resting and dead cells displayed small levels of cholesterol removal, compared with growing cells, ranged from 110.67 to 167.03 µg/mL.

Table 2

Cholesterol removal by growing, resting and heat–killed cells of lactic acid bacteria.

Strains	Assimilation of cholesterol (µg/mL)		
	Growing cells	Resting cells	Heat–killed cells
BCRC17474	26.58±0.16 ^a	6.35±0.22 ^b	6.54±0.36 ^b
BCRC17010	81.36±3.24 ^a	32.33±0.06 ^b	31.45±0.00 ^b
B0006	153.40±8.74 ^a	5.47±0.60 ^b	7.94±0.60 ^b
B0007	167.03±8.20 ^a	35.44±0.05 ^c	54.21±0.01 ^b
B0009	110.67±10.93 ^a	22.68±0.17 ^b	26.46±0.17 ^b
B0010	113.09±5.53 ^a	9.39±0.41 ^b	13.59±0.41 ^b
B0011	116.12±6.58 ^a	10.33±0.26 ^b	13.31±0.26 ^b
B0012	134.91±2.73 ^a	26.67±0.01 ^c	32.46±0.02 ^b
B0015	134.61±31.37 ^a	6.46±0.47 ^b	4.53±0.47 ^b
B0021	113.70±5.92 ^a	6.94±0.92 ^b	7.87±0.92 ^b
B0022	154.00±7.27 ^a	25.62±0.02 ^b	–27.37±0.05 ^c
B0023	131.27±2.73 ^a	1.40±0.01 ^c	16.67±0.01 ^b
B0025	130.36±7.77 ^a	6.32±0.28 ^b	6.64±0.28 ^b

BCRC17474: *L. johnsonii*; BCRC17010: *L. acidophilus*. The data are expressed as the mean±SD ($n=3$). Values in a row with different superscript letters are significantly different ($P<0.05$).

Table 3 shows the effects of acid on the viability of selected strains, B0006, B0007 and B0022. Although all strains

were sensitive to pH 2.0 after 3 h incubation (data not shown), all strains showed tolerance to pH 3.0 for 3 h. Strain B0007 was the highest acid tolerance, followed by strain B0022 and B0006. These findings suggest that B0006, B0007 and B0022 are acid tolerant strains, which they can survive well at pH 3.0.

Table 3

Effects of pH on viability of lactic acid bacteria.

Strains	Before treatment	pH 3.0
	log CFU/mL	log CFU/mL
BCRC17010	8.210±0.209 ^a	8.200±0.020 ^a
BCRC17474	9.710±0.176 ^a	9.640±0.044 ^a
B0006	9.490±0.020 ^a	8.440±0.056 ^b
B0007	9.730±0.075 ^a	8.800±0.015 ^b
B0022	9.470±0.133 ^a	8.640±0.119 ^a

BCRC17474: *L. johnsonii*; BCRC17010: *L. acidophilus*. The data are expressed as the mean±SD ($n=3$). Values in a row with different superscript letters are significantly different ($P<0.005$).

Table 4 shows the effects of different concentrations of bile salt on viability of strains tested. Growth of lactobacilli in MRS broth without bile salt was used as a control. All strains showed good growth in MRS broth without bile salt, whereas the growth of the strains decreased in the presence of 0.5% and 1% oxigall, compared with the control. However, the strains were found to be tolerant to bile salt, with 10^7 – 10^8 CFU/mL after incubation at 0.5% and 1% oxigall. This finding suggests that B0006, B0007 and B0022 were bile-tolerant strains.

Table 4

Effects of different concentration of bile salt on the viability of lactic acid bacteria.

Strains	0% bile salt	0.5% bile	1.0% bile
	log CFU/mL	log CFU/mL	log CFU/mL
BCRC17010	8.21±0.21 ^a	8.27±0.02 ^a	8.16±0.12 ^a
BCRC17474	9.64±0.26 ^a	8.24±0.04 ^b	8.24±0.24 ^b
B0006	9.49±0.02 ^a	7.58±0.08 ^b	7.52±0.17 ^b
B0007	9.73±0.08 ^a	8.07±0.01 ^b	7.90±0.10 ^b
B0022	9.47±0.13 ^a	7.54±0.05 ^b	7.91±0.02 ^b

BCRC17474: *L. johnsonii*; BCRC17010: *L. acidophilus*. The data are expressed as the mean±SD ($n=3$). Values in a row with different superscript letters are significantly different ($P<0.05$).

Table 5 shows adhesion of selected strains to the Caco–2 cell lines. Strain B0007 and B0022 showed strong adherence, compared to the reference strain BCRC 17474 and BCRC 17010. However, B0006 showed non adhering capability in this system. This observation revealed that the residence of strain B0007 and B0022 in the intestine may help establish and stabilize the microflora, thereby leading to cholesterol removal.

According to the experimental results, strain B0006, B0007 and B0022 were identified as *Pediococcus acidilactici*, *L. plantarum* and *L. plantarum* using 16S rDNA analysis, respectively. To confirm these findings, strain B0006, B0007 and B0022 were amplified and their 16S rDNA sequences were analyzed. The data obtained revealed that 16S rDNA sequence of strain B0006, B0007 and B0022 matched well with that of API 50 CHL and 16S rDNA nucleotide sequence of strain

B0006, B0007 and B0022 were 99% similarity with that in the GenBank.

Table 5

Adhesion of LAB strains to Caco–2 cell–line at normal state.

Strains	CFU*
BCRC17474	51±7 ^b
BCRC17010	17±11 ^a
B0006	13±6 ^a
B0007	223±2 ^c
B0022	271±57 ^d

BCRC17474: *L. johnsonii*. BCRC17010: *L. acidophilus*. *: Expressed as the average number of LAB adhering to human intestine cells, counts is carried out on 20 randomized microscopic fields. Values in a column with different superscript letters are significantly different ($P<0.05$).

4. Discussion

Recent studies have reported that administration of probiotics and prebiotics are effective in improving lipid profiles such as the reduction of serum total cholesterol, triglycerides, and low-density lipoprotein-cholesterol[7,13]. Reduction in total cholesterol and low-density lipoprotein cholesterol in hypercholesterolemic men may reduce the incidence of cardiovascular disease. Supplementation of diet with LAB-containing dairy foods may decrease serum cholesterol concentrations[1].

The possible mechanisms for removal of cholesterol by probiotics are proposed: assimilation of cholesterol during growth, incorporation of cholesterol into the membrane of cells, and binding of cholesterol to the cell surface[1]. It is well known that deconjugated bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids[9]. In the present study, all selected strains displayed higher cholesterol removal, compared with the reference strains. This means that assimilation of cholesterol by selected strains tested would make cholesterol less available for absorption into the circulation[3]. Especially, of the strains, strain B0006, B0007 and B0022 removed the highest level of cholesterol (more than 153 µg/mL), implying that strain B0006, B0007 and B0022, have high potential to remove cholesterol. As revealed in Table 2, even dead or resting cells still displayed little cholesterol assimilation. This removed activity of dead and resting cells on cholesterol implied that cholesterol may be removed through binding to cells[9]. In addition, based on the results, cholesterol removal was associated well with the bacterial growth. Cholesterol assimilation by growing cells was significantly higher than resting and dead cells, suggesting that the difference in the levels of cholesterol removed among the resting, dead cell and growing cells, was due to the uptake of cholesterol by the growing cells. This observation seems that growing cells can remove cholesterol from media both by binding of cholesterol to living cells and by uptake of cholesterol into living cells during growth[1].

Probiotics are live microorganisms that confer health on the host^[14]. Many bacteria such as lactobacilli and bifidobacteria are commonly used as probiotics. However, the microbes that are not native to intestinal organs can't live in a medium containing high acidity. Therefore, probiotics must overcome a hostile environment such as in the human gastrointestinal tract before benefits of them are available^[5,15]. In addition, stresses to organisms begin in the stomach, which pH between 1.5 and 3.0, and in the upper intestine that contains bile^[9]. Thus, it is necessary to test acid and bile tolerance of the strains tested. In the present work, in order to evaluate whether the selected strains are acidity and bile tolerance, the survival of the strains tested was investigated under various pH and bile treatments. According to the results from Table 3, a reduction in total colony-forming units was found in the selected strains, B0006, B0007 and B0022 after incubation at pH 2.0 for 3 h, compared with the control. The three selected strains decreased by 0.83 to 1.05 log cycles after incubation at pH 3.0 for 3 h. However, B0006, B0007, and B0022 exhibited the survival ratio of 89.12%, 90.44%, and 91.24%, in pH 3.0 treatment, respectively. Clearly, B0006, B0007, and B0022 displayed a significant acid tolerance. The finding is meaningful because the strains resistant to acidity are crucial for the manufacture of fermented foods and provide health as well.

Bile salt tolerance is considered one of the essential properties required for LAB to survive in the human intestine^[1,2,16]. The relevant physiological concentrations of human bile ranged from 0.3% to 0.5%^[17]. All selected strains showed better growth in MRS broth without bile compared to the reference strains. All strains showed similar growth in the presence of bile salt (0.5%–1.0%). From Table 4, the reduction in total colony-forming units was 1.66–1.93 log cycles and 1.56–1.97 log cycles after incubation at 0.5% and 1.0% of bile salt, respectively, compared with the control without bile. However, as revealed in Table 4, each strain exhibited the survival activity, even in 1.0% bile salt. Survival at pH 3 for 1.5 to 2 h is considered one standard for low-pH tolerance of probiotic bacteria^[1]. It is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile. Strains B0006, B0007, and B0022 all had strong resistance even in the presence of 1% bile salts. The ability to uptake cholesterol in laboratory culture media has been shown for numerous lactobacilli isolated from fermented mustard.

In order to select the strains for use microbiotic supplements, it is necessary to test the ability to adhere to mucosal surfaces in the human gastrointestinal tract. In the present work, strain B0007 and B0022 showed noticeable adherence into the Caco-2 cells. However, strain B0006 was found very weak adherence to the cells. Many reports suggested that retention in the intestinal tract is dependent

on adsorption of the bacteria to epithelial surfaces^[18]. We speculate that strain B0007 and B0022 have marked ability to adsorb to intestinal surfaces. This physical association with epithelial surfaces is an important determinant for subsequent cholesterol assimilation by growing cells.

Lactobacilli with probiotic characteristics isolated from traditionally homemade koumiss, *Lactobacillus* strains were able to lower cholesterol *in vitro*^[11]. Moreover, *L. plantarum*, a normal resident of the human gut microflora, is able to adhere to the epithelial cells, with a preference for the small intestine^[18]. In the present work, the results showed that strain B0007 and B0022 belonging to *L. plantarum* displayed marked cholesterol-lowering property and were able to adhere to the epithelial cell.

In conclusion, this work shows strain B0006, B0007 and B0022, isolated from fermented mustard, with remarkable cholesterol-lowering activity are acid and bile salt tolerance. In particular, strain B0007 and B0022 displayed strong adherence to Caco-2 cells. Considering the adherent activity, we suggest that strains B0007 and B0022 identified as *L. plantarum*, may be potential candidates for supplement to lower serum cholesterol. However, further *in vivo* verification is necessary.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Overmuch cholesterol in the blood is a major risk factor for coronary heart disease. Therefore, reduction of excess cholesterol is an important issue. Many kind of *Lactobacillus* cultures have been proven to exert potential hypocholesterolemic activity. It is needed to screen lactobacilli with probiotic characteristics and to determine the effect of the screened *Lactobacillus* strains for their ability to lower cholesterol.

Research frontiers

The ability of 50 LAB strains isolated from fermented mustard on lowering cholesterol *in vitro* was determined. Strain B0007, B0006 and B0022 assimilated more cholesterol

than BCRC 10474 and BCRC 17010. The strains of B0007 and B0022 may be potential functional sources for cholesterol-lowering activities as well as adhering to Caco-2 cell lines.

Related reports

The reduction of serum cholesterol could be an important health benefit of LAB, demonstrated in human, mouse, pig studies and rats. Consumption of dairy products containing probiotics has been proposed as a mean to lower serum cholesterol. Many kind of *Lactobacillus* cultures exerted potential hypocholesterolemic activity.

Innovations and breakthroughs

The aim of this study was to screen lactobacilli with probiotic characteristics isolated from traditionally fermented mustard, and to determine the effect of the screened *Lactobacillus* strains for their ability to lower cholesterol and the strains were also identified.

Applications

From the literature survey it has been found that LAB are generally recognized as safe (GRAS). Probiotic cultures of lactobacilli have potential health benefits. Several studies indicate consumption of certain cultured dairy products could lower total plasma cholesterol and low-density lipoprotein cholesterol.

Peer review

This work shows strain B0006, B0007 and B0022 with remarkable cholesterol-lowering activity are acid and bile salt tolerance. In particular, strain B0007 and B0022 displayed strong adherence to Caco-2 cells. Considering the adherent activity, we suggest that strains B0007 and B0022 may be potential alternatives for supplement to lower serum cholesterol.

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